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A Colorimetric Micro-method for the Determination of Glycogen in Tissues

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A new method for the determination of blood sugar has recently been described by Mendel & Hoogland (1950) and Mendel, Kemp & Myers (1954). This method is based on a colour reaction which occurs when a dilute solution of glucose is heated with concentrated sulphuric acid (Mendel & Bauch, 1926). Since glycogen is hydrolysed to glucose in hot sulphuric acid, this reaction can also be used to determine glycogen.

The deproteinizing solution used by Mendel & Hoogland (1950) contains trichloroacetic acid and, in addition, a small amount of silver sulphate to precipitate free chloride and other interfering substances. Although pure glycogen dissolves readily in this deproteinizing solution, only part of the glycogen can be extracted from the tissues with a cold solution of trichloroacetic acid (Willstätter & Rhodewald, 1934; Young, 1937; Bloom, Lewis, Schumpert & Shen, 1951; cf. Przylecki & Majmin, 1934; Mystkowski, 1937). However, it was found in the present investigation that all of the glycogen can be brought into solution by grinding the tissue with trichloroacetic acid solution and then heating the suspension for 15 min. at 100°.

Any glucose present in the tissues will of course be extracted and determined, together with the glycogen by this method. The amount of glucose in the tissues is usually small in comparison with the amount of glycogen present, but under certain experimental conditions it might be desirable to remove the glucose. Procedures will, therefore, be described for the determination of both glycogen and glucose in muscle and liver. Of the glycogen metabolites containing a hexose molecule, only glucose 1-phosphate gives the colour reaction (Mendel *et al.* 1954); however, the concentration of glucose 1-phosphate in tissues (cf. Umbreit, Burris & Stauffer, 1945) is within the limits of error of this determination.

METHODS

Reagents

Deproteinizing solution. Trichloroacetic acid (5 g., A.R.) and Ag_2SO_4 (100 mg., A.R.) are dissolved in water and made up to 100 ml. (cf. Mendel *et al.* 1954). The solution is stored in an amber bottle in the cold.

Sulphuric acid, 96% (w/w, approx. 36N) (cf. Kemp & Zuidweg, 1952; Mendel *et al.* 1954).

Methanol, 80% (v/v).

Procedure

Determination of glycogen plus glucose. Muscle or liver (25–75 mg.) are ground with 5 ml. of the deproteinizing solution in a centrifuge tube; a stainless-steel pestle with longitudinal grooves which fits closely into a cylindrical centrifuge tube has proved useful for this purpose. The fluid level is marked on the centrifuge tube and the tube, covered with a glass cap, is placed in a boiling-water bath for 15 min. Then the tube is cooled in running water, filled up to the mark with deproteinizing solution to compensate for evaporation, and centrifuged at 3000 rev./min. for 5 min. One ml. of the clear supernatant fluid is added to 3 ml. of H_2SO_4 in a wide test tube and mixed by vigorous shaking. The mixture is heated in a boiling-water bath for exactly 6·5 min. and subsequently cooled in running tap water. The intensity of the pink colour produced is measured spectrophotometrically at 520 mμ. and the glycogen concentration read from a standard curve in terms of glucose equivalents. As noted previously (Mendel *et al.* 1954), glycogen gives the same colour intensity as does an equivalent amount of glucose.

The intensity of the pink colour is proportional to the amount of glycogen up to a concentration of 150 μg. glycogen/ml. extract. Should the amount of glycogen in the tissues be so high that the glycogen concentration in the deproteinized extract is greater than 150 μg./ml., as will usually be the case with liver, the extract should be diluted before the colour reaction is carried out. A convenient dilution for normal liver is such that 1 ml. of the deproteinized solution corresponds to 2 mg. of fresh liver tissue.

In view of the uneven distribution of the glycogen in muscle, an alternative procedure may be used in order to obtain better average values. Larger pieces of muscle weighing 200–250 mg. can be extracted with 5 ml. of the deproteinizing solution as described above. However, after heating and restoring the suspension to its original volume, another 5 ml. of the deproteinizing solution should be added to remove any remaining chloride.

Determination of glucose and glycogen separately. The tissue sample is ground with 5 ml. of 80% (v/v) methanol. The suspension is centrifuged and the supernatant fluid containing the glucose is decanted into a calibrated centrifuge tube. Approximately 10 mg. of powdered charcoal are added to this methanol solution; the charcoal does not adsorb any hexose present, but will remove organic substances which would otherwise interfere with the colour reaction. The methanol is now removed completely under reduced pressure while heating the tube in warm water. Deproteinizing solution is added to the residual aqueous solution, still containing the charcoal, to bring the total volume to 5 ml. The suspension is centrifuged and the colour reaction is carried out with 1 ml. of the clear supernatant fluid, as described above. The amount of glucose extracted from the tissue sample in 80% (v/v) methanol can be estimated from the intensity of the pink colour produced.

Since glycogen is insoluble in 80% (v/v) methanol, the glycogen present in the original sample of tissue can be recovered from the precipitated residue remaining after extraction of the glucose with methanol. This tissue residue can be suspended in 5 ml. of deproteinizing fluid, the glycogen extracted by heating at 100° for 15 min. and the amount of glycogen determined as described above.

RESULTS

The results obtained are given in Tables 1 and 2. In order to compare these results with those obtained by the classical methods, the glycogen was also isolated according to Pflüger (cf. Good, Kramer & Somogyi, 1933), dissolved in water, and determined by the colorimetric method (Mendel *et al.* 1954). It can be seen from Tables 1 and 2 that the two methods give very similar values for the glycogen content of tissues. The amount of free glucose in the tissues is small (Table 2) and can usually be neglected when the glycogen content of normal muscle or normal liver is being estimated.

Table 1. *Determination of glycogen in rat gastrocnemius muscle with the new micro-method and with a modification of Pflüger's method*

The glycogen in the gastrocnemius muscle of normally fed rats was extracted with trichloroacetic acid as described in the text, or by a modification of Pflüger's method; in both cases, the amount of glycogen was determined colorimetrically as described by Mendel *et al.* (1954). The glycogen content of the fresh muscle was calculated in terms of glucose equivalents. Mean values are given ±standard error; when only two determinations were made the individual results are given.

	New micro-method			Pflüger's method		
Rat no.	No. of determinations	Wt. of muscle samples (mg.)	Glycogen content (%)	No. of determinations	Wt. of muscle samples (mg.)	Glycogen content (%)
1	5	273–482	0·49±0·017	2	941, 1038	0·54, 0·56
2	5	182–221	0·40±0·009	2	807, 914	0·36, 0·36
3	6	182–215	0·43±0·012	2	979, 1102	0·42, 0·44
4	5	55–79	0·32±0·017	2	1331, 1645	0·35, 0·39
5	7	24–41	0·58±0·015	5	551–926	0·61±0·017

Table 2. *Determination of glycogen and glucose in the liver and gastrocnemius muscle of the rat*

The glycogen and glucose were extracted from the tissues of well-fed animals by both of the procedures described in the text. The carbohydrate extracted from fresh tissue with hot trichloroacetic acid represents glycogen plus glucose; the methanol-soluble fraction represents glucose, while the methanol-insoluble fraction represents glycogen. For purpose of comparison, the glycogen was also isolated from the same tissues by a modification of Pflüger's method. In all cases the amount of carbohydrate was determined colorimetrically as described by Mendel *et al.* (1954) and expressed as glucose equivalents. Mean values are given ±standard error; when only two determinations were made the individual results are given.

		New micro-method				Pflüger's method		
Rat no.	Tissue	No. of determinations	Wt. of tissue samples (mg.)	Glycogen plus glucose (%)	Glucose (%)	Glycogen (%)	No. of determinations	Wt. of tissue samples (mg.)	Glycogen (%)
6	Liver	3	26–73	2·12±0·011	0·44±0·022	1·76±0·05	2	331–396	1·73, 1·61
7	Muscle	3	27–54	0·54±0·011	0·09±0·003	0·45±0·011	3	635–850	0·51±0·017

DISCUSSION

The new micro-method for determination of glycogen in tissues gives reasonably accurate results with 25–75 mg. of muscle or 10 mg. of liver. These results are in good agreement with those obtained by Pflüger's method of extraction; however, the new method has several advantages. In the first place, the glycogen content of a small piece of tissue can be readily determined. Fifteen to twenty determinations can be done on a single rat muscle weighing 2 g.; this would make it possible, for example, to determine the distribution of glycogen within the muscle itself. Similarly, the small amount of liver tissue necessary for the determination would allow the estimation of glycogen in biopsy specimens. Furthermore, the new method is simpler and more rapid than the classical methods; the glycogen content of twenty tissue samples can be determined in about 2 hr.

The procedures described above are concerned only with the determination of the total glycogen content of tissues. It seems possible, however, that two different forms of glycogen may exist in the tissues. As noted previously, only part of the glycogen can be extracted from the tissues with cold trichloroacetic acid, and it is necessary to heat the suspension or to destroy the tissue by alkaline hydrolysis before the remaining glycogen can be brought into solution. The results obtained by Bloom *et al.* (1951) and by Bloom & Knowlton (1953) seem to indicate that the relationship between these two glycogen fractions in muscle can be altered by subjecting the animal to various experimental conditions. The new micro-method for the determination of glycogen could easily be adapted to a study of this problem by carrying out the extraction with deproteinizing solution in two stages, that is, first in the cold and subsequently at 100°.

SUMMARY

1. A simple micro-method for the determination of glycogen in tissues has been described. The tissue is extracted with a solution of trichloroacetic acid at 100° and the glycogen in the extracts is determined, without previous hydrolysis, by the colorimetric method described by Mendel *et al.* (1954).

2. A procedure is described by which the glucose can be extracted from the tissue and determined separately.

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Spectrophotometric Studies of the Reaction of Methaemoglobin with Hydrogen Peroxide

1. THE FORMATION OF METHAEMOGLOBIN-HYDROGEN PEROXIDE

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The unstable red substance formed when hydrogen peroxide is added to an acid methaemoglobin solution (Kobert, 1900) was shown by Keilin & Hartree (1935) and Haurowitz (1935) to be a well-defined compound apparently analogous to the azide, fluoride, cyanide and hydrosulphide compounds of methaemoglobin, with absorption bands at 545 and 589 mμ., and requiring for its formation 1 molecule of hydrogen peroxide for each iron atom of haematin. The compound is unstable, and in the subsequent reaction part of the methaemoglobin is recovered whilst the remainder is lost by oxidative degradation to unidentified products (Keilin & Hartree, 1935).

We have shown that in the reaction between human haemoglobin and hydrogen peroxide in the